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Engrailed, a homeodomain protein, can repress *in vitro* transcription by competition with the TATA box-binding protein transcription factor IID

(general transcription factors/*Drosophila*/*Hsp70* promoter/ DNase I footprinting)

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ABSTRACT **Engrailed (En) is a homeodomain protein that binds to a consensus sequence (NP) and plays an important role during *Drosophila* development. Purified En, which is produced in *Escherichia coli*, binds not only to this consensus sequence but also to the TATA box of the *Drosophila Hsp70* promoter and of other eukaryotic promoters. Interestingly, En represses transcription of these promoters in an *in vitro*-reconstituted mammalian transcription system and footprint analyses show that En competes with the TATA box-binding protein transcription factor IID for binding to the TATA box. In contrast, a stable template-committed complex formed by preincubation of transcription factor IID with the promoter is not disrupted by addition of En, and in this case transcription is not repressed. These *in vitro* studies suggest a transcriptional repression mechanism, involving competition between En and transcription factor IID for TATA box binding, that may be involved in En-mediated repression *in vivo*.**

The sequential activation of a hierarchy of regulatory genes plays an important role in the precise temporal and spatial pattern of *Drosophila* development (1, 2). More than 40 such developmental genes have been identified in *Drosophila*. Many of them have been cloned and more than half of the characterized genes are related by a region of sequence homology called the homeobox, which encodes the homeodomain (3, 4). The homeodomain contains a region similar to the helix-turn-helix motif present in many prokaryotic DNA binding proteins (5, 6) and several *Drosophila* homeodomain-containing proteins expressed in *Escherichia coli* from cloned cDNAs have been shown *in vitro* to have sequence-specific DNA binding properties (7–9). Several mammalian transcription factors have also been shown to contain homeodomains, although these diverge markedly from characterized homeodomain sequences (for review, see ref. 10). These observations suggest that the *Drosophila* homeodomain proteins are transcription factors whose function is to modulate gene expression during development by site-specific DNA interactions.

The *Drosophila* segment-polarity gene engrailed is one of the homeodomain-encoding genes necessary for the proper determination of the segmental subdivisions of the *Drosophila* embryo. The engrailed gene product (En) expressed in *E. coli* binds to the consensus sequence TCAATTAAAT (NP), which is found in clusters in the engrailed regulatory region (8, 9). We will call this sequence the En binding site although several other homeodomain proteins [fushi tarazu (Ftz), even-skipped (Eve), and zerknüllt (Zen)] also recognize it (8, 9). Transient expression experiments in *Drosophila* cell lines have shown that the addition of specific homeodomain protein binding sites to a promoter allows its regulation by the corresponding protein (11, 12). En can repress transcription from a promoter containing En binding sites when this transcription is activated by other homeodomain proteins such as Ftz or Zen (11, 12). In these cell culture experiments, both activation and repression by the homeodomain proteins are dependent on the presence of En binding sites.

To identify any direct effect of En on transcriptional repression and to examine its molecular mechanism, we used an *in vitro* transcription system. Our results indicate that En can act as a transcriptional repressor by directly inhibiting the formation of the preinitiation complex.

MATERIALS AND METHODS

Recombinant Plasmids. The pAR-engrailed expression plasmid was constructed by Hoey and Levine (9). The plasmid HZ50pL contains *Hsp70* DNA from positions −50 to +271 from the cap site (13). The plasmid NP6-HZ50pL contains six repeats of the En consensus sequence (NP6) (8) inserted into the *Kpn* I–*Xba* I site of HZ50pL (11). The plasmid pMLH1 is described in Hawley and Roeder (14). This plasmid contains the adenovirus 2 major late (ML) promoter and 536 base pairs of downstream sequences (15). The plasmid 5′Δ-40 contains *ftz* DNA from positions −40 to +73 from the transcription start site (16). The plasmid NP6-5′Δ-40 contains the NP6 sequence in the *Xba* I site of 5′Δ-40 (J.-P. Vincent and P. O'Farrell, personal communication).

Purification of En. The En-expressing cell extract was prepared essentially as described (17). ^{35}S-labeled En was also prepared as a marker for the purification (18). A cell extract (50 mg of protein) containing 2 μCi (1 Ci = 37 GBq) of ^{35}S-labeled En was used as starting material. En was purified on heparin-agarose (Bio-Rad), followed by DEAE-cellulose (DE52; Whatman), a mutant oligonucleotide affinity column, and finally a specific oligonucleotide (NP3) column, as described (unpublished data). En (200 μg) was obtained after the final column.

DNase I Footprinting Assay with Purified En. About 500 ng of DNA fragments were end-labeled with [α-^{32}P]dATP, [α-^{32}P]dCTP, and the Klenow fragment of DNA polymerase I. Each probe (20 fmol, 5 ng) was incubated with various amounts of En in 20 mM Hepes, pH 7.8/65 mM KCl/2 mM $MgCl_2$/poly(dI-dC)·poly(dI-dC) (4 μg/ml)/bovine serum albumin (0.1 mg/ml) for 30 min at 30°C. DNase I (2 μl at 20 μg/ml) was added and incubated for 30 sec at 30°C. The reaction was stopped by the addition of 50 μl of stop buffer [20 mM EDTA/0.6 M sodium acetate, pH 5.2/0.2% SDS/yeast tRNA (100 μg/ml)]. The DNA was isolated by phenol/chloroform and chloroform extractions followed by ethanol

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Abbreviations: ML, major late; TFIID, TFIIB, etc., transcription factor IID, transcription factor IIB, etc.

precipitation. The DNA was then analyzed on an 8% sequencing gel. For reactions with transcription factor IID (TFIID) (see Figs. 2*B* and 3*B*), the binding reaction components were slightly different: 12 mM Tris·HCl, pH 7.7 at 4°C/4 mM $MgCl_2$/60 mM KCl/40 mM Hepes, pH 8.4/poly(dG-dC)·poly(dG-dC) (2 μg/ml)/bovine serum albumin (0.1 mg/ml)/0.6 μg of ω-aminooctyl TFIID (22, 26) were used for these reactions.

***In Vitro* Transcription.** Transcription reactions were performed essentially as described (19), except that 3 mM $MgCl_2$ and 600 ng of supercoiled template DNA were used. The amounts of general transcription factors for transcription were the same as in Hai *et al.* (20). After incubation at 30°C for 60 min, transcription was stopped by adding 75 μl of 450 mM sodium acetate, pH 5.2/10 mM EDTA/0.5% SDS/yeast tRNA (50 μg/ml) to the reaction mixture. Primer-extension analysis of each transcript was done as described (21). A 21-mer synthetic oligonucleotide (5′-GGTTGATTTCAG-TAGTTGCAG-3′) was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as a primer. The products were analyzed on a denaturing 6% polyacrylamide gel. For the run-off assay, transcription reactions were performed in the same way as above except for the GTP concentration and the template DNA: 25 μM GTP and 5 μM [α-^{32}P]GTP were added and linear templates were used for these reactions.

RESULTS

Interaction of En with the *Drosophila Hsp70* Promoter. To determine whether En can act directly on the promoter to repress transcription, we purified En that had been overproduced in *E. coli* and carried out a DNase I footprinting analysis to examine the interactions of En with the *Drosophila Hsp70* promoter with (NP6-HZ50pL) or without (HZ50pL) upstream En binding sites (Fig. 1*C*). En protected not only the six En binding sequences of NP6-HZ50pL but also the TATA box and other downstream sequences (hI–hVI) that match the NP sequence (Fig. 1*A*). A strong DNase I-hypersensitive site was detected on both sides of the region of the TATA box protected by En in NP6-HZ50pL and HZ50pL (at positions −37 and −14). The binding affinity of En was different for each region (Fig. 1): NP6 and hVI were completely protected by 40 ng of En whereas the TATA box and sites hI–hV needed 225 ng of En for complete protection. The binding of En to the TATA box and the downstream sites of HZ50pL was not dependent on the presence of the NP sites (Fig. 1*B*). These results suggested the possibility that En might act as a transcriptional repressor by competition with the TATA box-binding protein TFIID for binding to the TATA box.

En Represses Transcription of the *Hsp70* Promoter *in Vitro*. To test this possibility, we examined transcription of the *Drosophila Hsp70* promoter in a reconstituted *in vitro* system containing the general transcription factors (TFIIB, TFIID, TFIIE, and RNA polymerase II) (for review, see ref. 22) plus various amounts of En. Fig. 2*A* shows that 400 ng of En was sufficient to repress transcription of the *Hsp70* promoter more than 10-fold in the presence or absence of the NP6 upstream sequence (NP6-HZ50pL and HZ50pL, respectively). This correlates with the amount of En protein required for complete protection of the TATA box from DNase I since twice as much DNA (60 fmol) was used in the transcription reaction mixtures, making the molar ratio of En protein to En binding sites ≈10 in each experiment.

To examine how En and TFIID interact with the *Drosophila Hsp70* promoter, the DNase I footprints of En and TFIID on these constructs were compared (Fig. 2*B*). A HeLa cell fraction containing TFIID showed the characteristic pattern of interaction, extending from positions −47 to +25 (22). This pattern could easily be distinguished from the En footprint on the TATA box, which exhibited immediately flanking hypersensitive sites (Fig. 2*B*). In contrast to En,

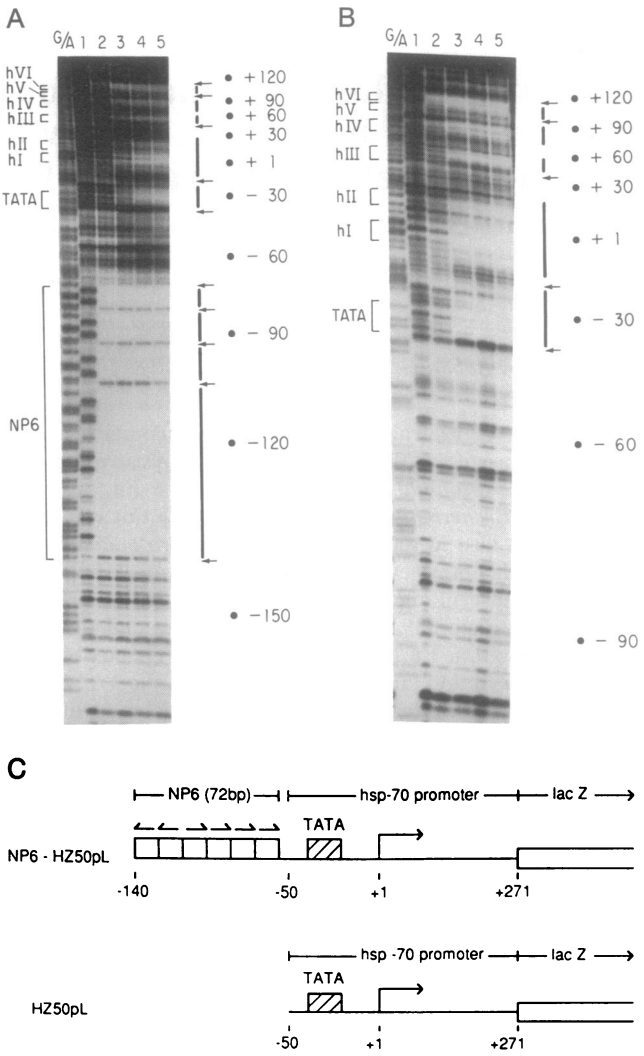

FIG. 1. Interaction of En with the *Hsp70* promoter. DNase I footprinting reactions were performed by using two *Hin*dIII–*Xmn* I fragments of NP6-HZ50pL and HZ50pL (labeled at the 3′ end of the transcribed strand prior to footprinting), as described in *C*. (*A*) NP6-HZ50pL fragment (positions −175 to +209). (*B*) HZ50pL fragment (positions −110 to +209). G+A sequencing reactions were electrophoresed in adjacent lanes. Amounts of En used are as follows. Lanes: 1, no protein; 2, 40 ng; 3, 120 ng; 4, 225 ng; 5, 400 ng. The protected regions are indicated by thick lines, and the hypersensitive sites are indicated by arrows. The location of the TATA box, NP6 sites, and other En binding sites are indicated. The En binding sites other than NP6 and the TATA box are designated by numbers hI–hVI, starting from the site closest to the TATA box.

TFIID did not bind to the NP6 sequence. Because we could distinguish between the En and TFIID patterns of protection, we used a combination of both proteins to footprint the NP6-HZ50pL or HZ50pL constructs. The patterns of protection observed on the TATA box regions were the same for each promoter and showed only the footprint profile characteristic of En (Fig. 2*B*).

Binding of En to the TATA Box Is Important for the Repression of Transcription by En. These DNase I footprinting analyses indicated that En bound not only to the TATA box but also to downstream sequences (hI–hVI; Fig. 1 *A* and *B*). Therefore, it was still possible that the downstream sites were involved in the repression.

TFIID can form a stable template committed complex with TATA box-containing promoters (23–25). We used this property of TFIID to identify the En binding sites essential for transcriptional repression of NP6-HZ50pL and HZ50pL. We

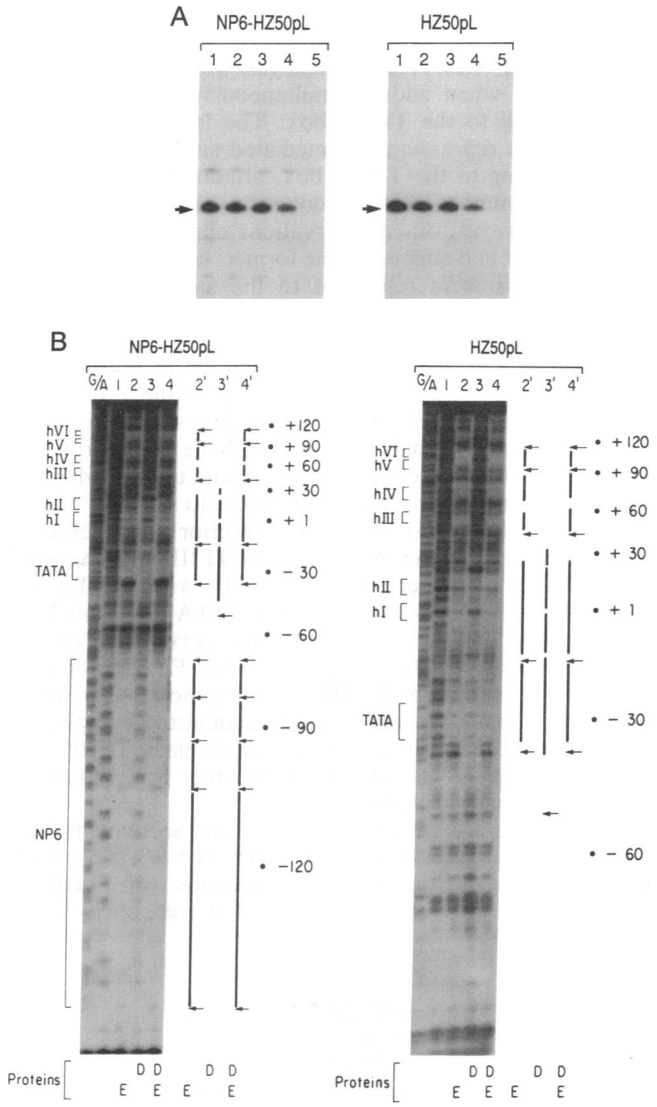

FIG. 2. Effect of En on *in vitro* transcription. (*A*) Primer-extension analysis was carried out in the reconstituted transcription system at various concentrations of En. NP6-HZ50pL (60 fmol) and HZ50pL (60 fmol) were used as templates. Amounts of En used are as follows. Lanes: 1, no protein; 2, 50 ng; 3, 100 ng; 4, 200 ng; 5, 400 ng. Arrows indicate the specific transcripts (170 nucleotides). (*B*) DNase I footprinting reactions were performed as in Fig. 1 to see the interactions of En and TFIID with the *Hsp70* promoter except that the amount of DNA fragments for each reaction was about 1 ng (4 fmol) and ω-amino octyl TFIID was used. The fragments used are as in Fig. 1. No protein was used in lane 1; TFIID was used in lanes 3 and 4 (lanes D). En (160 ng) was used in lanes 2 and 4 (lanes E). Protected regions, hypersensitive sites, and En binding sites are indicated in lanes 2′, 3′, and 4′, corresponding to lanes 2, 3, and 4.

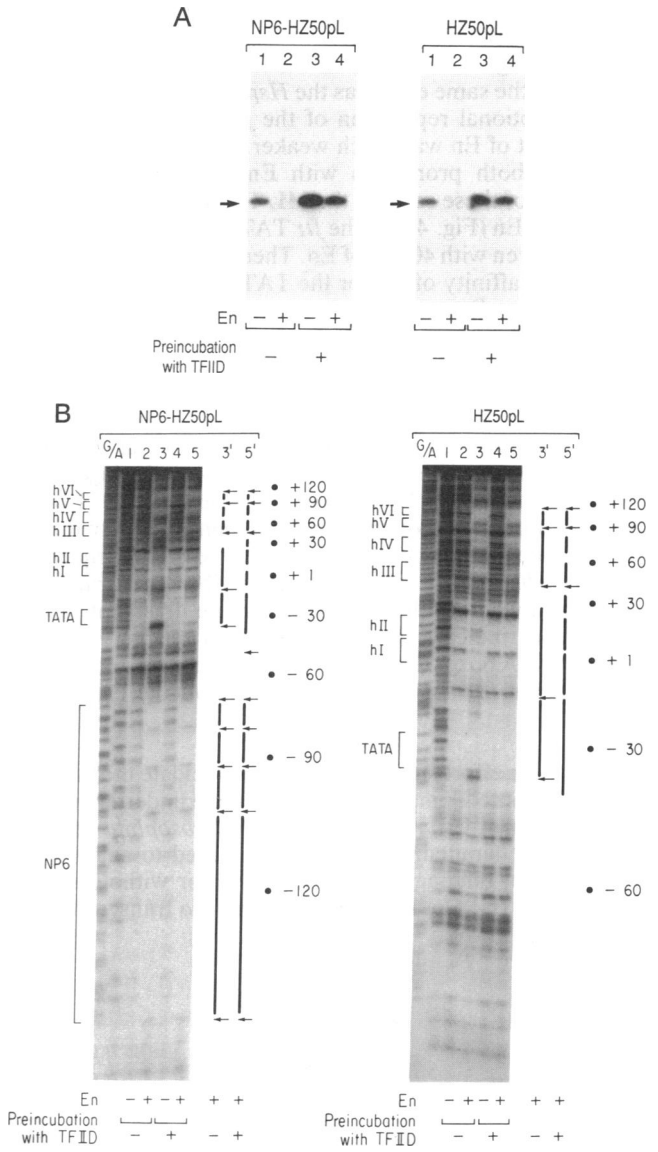

FIG. 3. Effect of En on transcription after TFIID–promoter preincubation. (*A*) Primer-extension analysis was carried out to examine the transcriptional repression by En. The same templates were used as in Fig. 2*A*. These templates were preincubated with or without TFIID for 30 min at 30°C, prior to addition of En and of the other factors (TFIIB, TFIIE, and RNA polymerase II). Lanes: 1 and 2, preincubation without TFIID; 3 and 4, preincubation with TFIID; 1 and 3, no En; 2 and 4, 400 ng of En. (*B*) DNase I footprinting reactions were carried out after preincubation with or without TFIID in the same conditions as in Fig. 2*B*. The same fragments as in Fig. 1 were used for footprinting analysis. Conditions are as follows. Lanes: 1, no protein added; 2 and 3, preincubation without TFIID and then addition of TFIID alone or TFIID plus 225 ng of En, respectively; 4 or 5, preincubation with TFIID without further addition of En or with addition of 225 ng of En after the preincubation, respectively. Protected regions, hypersensitive sites, and En binding sites are indicated in lanes 3′ and 5′, corresponding to lanes 3 and 5.

preincubated the two promoters with TFIID to form the complex; we then added En and the other general factors needed to start transcription and checked for any effect of En on the repression of transcription (Fig. 3*A*) and on the stability of the preformed TFIID–DNA complex (Fig. 3*B*). Contrary to what we observed when En and TFIID were added simultaneously (Fig. 2*A*), En repressed transcription by only 50% when it was added after preincubation with TFIID (Fig. 3*A*, lane 4). Similarly, after preincubation with TFIID (Fig. 3*B*, lane 5), En was almost unable to compete with TFIID for binding to the TATA box. It still bound to the same extent to the other regions (NP6 and hI–hVI). Thus, the resulting hybrid pattern showed interactions over positions −52 to −13 that were identical to those observed with TFIID alone (Fig. 3*B*, lanes 2 and 4), while the interactions over positions +30 to +120 and (when present) NP6 were the same as those observed with En alone. Thus, we can conclude that the repression observed *in vitro* results from a direct inhibition of TFIID binding.

Other TATA-Containing Promoters Can Be Repressed by En. To generalize our results to other genes and to analyze whether transcription of other TATA box-containing promoters is also repressed by En, we used the adenovirus 2 ML

and fushi tarazu (*ftz*) promoters as templates. En repressed transcription of these promoters but to very different extents (Fig. 4): 225 ng of En repressed the transcription of the ML promoter to the same extent as the *Hsp70* promoter, whereas the transcriptional repression of the *ftz* promoter with the same amount of En was much weaker. DNase I footprinting analysis of both promoters with En provides a possible explanation of these results: the ML TATA box is protected by 200 ng of En (Fig. 4) but the *ftz* TATA box is only weakly protected, even with 400 ng of En. There is a good correlation between the affinity of En for the TATA box and the extent of repression observed. The sequence of the TATA box appears to be important: both the *Hsp70* and ML promoters contain the same TATA box, TATAAA, but the *ftz* promoter contains a different sequence, TATATA (16).

To understand the reason for the recognition of the TATA box by En, we compared the sequences of all the sites protected by En. They all match a 9-base-pair degenerate consensus sequence, HCWATHAAA (where H is A, T, or C and W is A or T), although En has the highest affinity for the NP sequence that has been reported (8). The TATA sequences of the *Hsp70* and ML promoters match the degenerate consensus well whereas the *ftz* TATA is more divergent. These observations indicate that the strength of binding of En to various TATA boxes determines its *in vitro* repression activity that results from the exclusion of TFIID from the TATA box.

DISCUSSION

As part of our efforts to understand and elucidate the function of various homeodomain proteins, we have undertaken an *in vitro* analysis of En, the product of the *Drosophila* segmentation gene engrailed. Purified En was used to analyze the pattern of En binding to promoters with or without six En binding sites (NP6) and its effect on *in vitro* transcription of the same promoters. Somewhat surprisingly, En repressed transcription of the *Hsp70* promoter independently of the presence of NP sites. A further analysis with purified factors revealed that when added simultaneously, En prevented TFIID binding to the TATA box. The latter observation suggested that repression was mediated simply by preemptive En binding to the TATA box, although a mechanism involving En interactions at the downstream regions was also possible. However, other observations eliminated this second possibility and supported the former. (*i*) The *Hsp70* and ML promoters were repressed to the same extent even though the location of En binding sites downstream of the TATA box varies widely (see *Hsp70* and ML, Figs. 1 and 4). (*ii*) The degree of transcriptional repression of several promoters correlated with the affinity of En for the TATA box despite the presence of other strong binding sites in adjacent regions. Thus, En strongly repressed both the adenovirus ML and the *Hsp70* promoters to which it bound tightly, whereas it repressed only poorly the *ftz* promoter to which it bound very weakly. (*iii*) Most important, the prior formation of a template-committed complex between TFIID and the promoter (23–25) prevented repression by En without affecting its binding to any sites other than the TATA box. This lack of repression correlated well with the persistence of the TFIID footprint over the TATA box when En addition was preceded by binding of TFIID to the promoter. Our interpretation is that once the template-committed complex is formed, addition of En can no longer displace TFIID and cannot prevent the recruitment of the other general factors needed to start transcription.

In Fig. 5, we present a simple model that accounts for the transcriptional repression by En under our *in vitro* conditions. En works as a repressor by competition with TFIID for binding to the TATA box (Fig. 5*A*). However, once TFIID

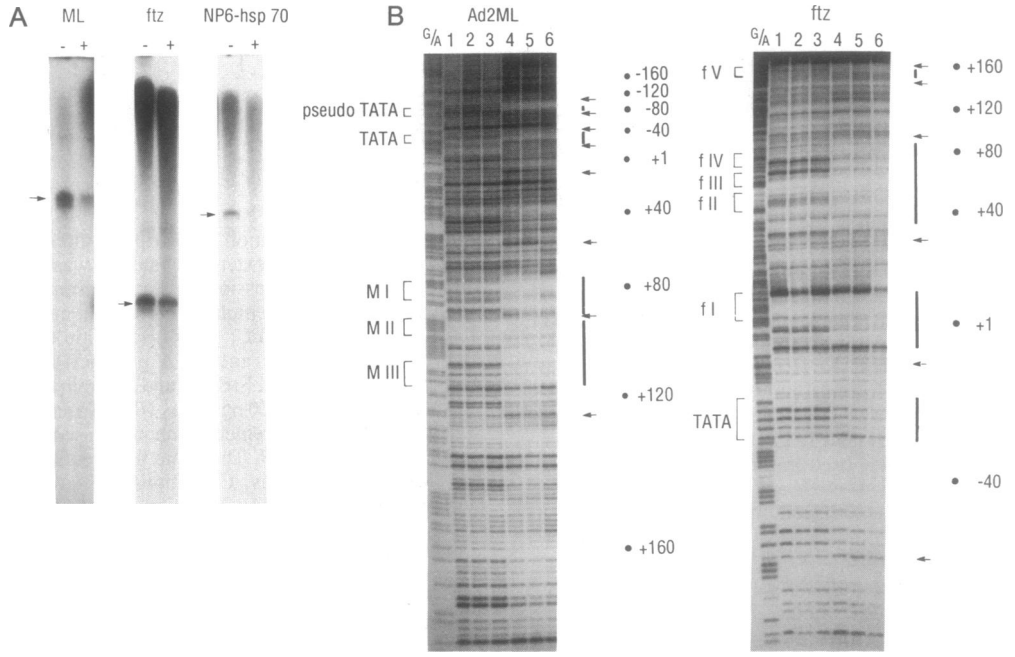

FIG. 4. Interactions of En with various promoters. (*A*) Effect of En on transcription of various promoters. Run-off assays were carried out using different promoters (30 fmol) with or without En. Lanes: ML, *Sma* I-digested pMLH1, which contains the adenovirus 2 ML promoter; ftz, *Sau* I-digested 5′Δ-40, which contains the *ftz* promoter; NP6-hsp-70, *Sau* I-digested NP6-HZ50pL; −, no En; +, 225 ng of En. Each specific transcript is shown by an arrow. (*B*) DNase I footprinting of various promoters. The reactions were performed as above except that 10 fmol of DNA was used. Lanes Ad2ML contain an *Xho* I–*Hin*dIII fragment of pSmaF, which contains 456-base-pair adenovirus 2 ML promoter. The fragment was labeled at the 3′ end of the nontranscribed strand. Amounts of En used are as follows. Lanes: 1, no protein; 2, 25 ng; 3, 50 ng; 4, 100 ng; 5, 200 ng; 6, 400 ng. Lanes ftz contain an *Xba* I–*Pvu* II fragment of 5′Δ-40 that contains the *ftz* promoter (ftz, 270 base pairs). The fragment was labeled at the 3′ end of the transcribed strand. All lanes are the same conditions as for lanes Ad2ML. The protected regions are indicated by thick lines and the hypersensitive sites are indicated by arrows. En binding sites are designated by numbers MI–MIII for the ML promoter and fI–fV for the *ftz* promoter.

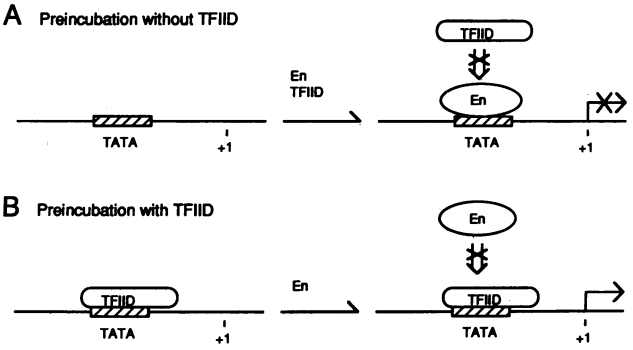

FIG. 5. Proposed model for transcriptional repression by En. (*A*) Preincubation without TFIID. When TFIID and En are added at the same time, En can compete out TFIID and bind to the TATA box. As a result, transcription is repressed. (*B*) Preincubation with TFIID. Preincubation allows the formation of a stable complex with the TATA box containing template and En cannot bind to the TATA box. In this case, En has no effect on transcription.

forms a stable complex with the promoter, En can no longer bind to the TATA box and cannot repress transcription (Fig. 5*B*). Models proposed for the activation mechanisms of transcription factors such as GAL4, activating transcription factor (ATF), upstream stimulatory factor (USF), and viral immediate early proteins also involve TFIID (20, 26–29). In these cases, protein–protein interactions between the activator proteins and TFIID are assumed to alter the binding of TFIID to the promoter in ways that lead to transcriptional activation. The results from transient-expression experiments in *Drosophila* cells led to a different interpretation from ours (11, 12). These studies demonstrated that transcription from promoters with En binding sites was enhanced by upstream homeodomain-containing activators that bound to these sites, and that coexpression of En suppressed this activation, leading to the simple model that En represses transcription by blocking binding of the activators. Indeed, we show elsewhere (unpublished data) that low concentrations of En selectively inhibited Ftz-dependent *in vitro* transcription activation by preventing Ftz binding, with no effect on basal activity and TFIID binding.

Thus, our combined *in vitro* results demonstrate two possible mechanisms for the suppression by En of the transcriptional activation mediated by site-specific binding proteins, such as Ftz or Zen. One mechanism would involve competition for binding to the NP sites upstream of the TATA box and the other would require more direct action on components of the general transcription machinery (see also ref. 30). The two models are not incompatible and in the normal *in vivo* situation, where the level of En may be lower, these two mechanisms might act in concert (e.g., by cooperative binding of En to the TATA box and to upstream sites). In fact, the failure to observe transcriptional repression by En in cell culture in the absence of En binding sites does not necessarily contradict the present data since basal promoter activity (in the absence of En binding sites) could not be accurately measured in those studies. In addition, since the reporter and En producer plasmids were cotransfected, the time lag before En expression could have been sufficient to allow formation of a stable preinitiation complex on the reporter gene, forestalling repression by En.

Although a repressive mechanism acting directly on the TATA box has a potentially broad spectrum, occupancy of the TATA box by TFIID prevents repression by this mechanism and may in turn be enhanced by other activators (see ref. 29). Regarding the potential *in vivo* relevance of the mechanism proposed here, a provocative observation is that engrailed, which is auto-activated, does not have a TATA box. This could explain why En does not repress its own expression even though there are En binding sites upstream of the engrailed promoter (12). Hence, whether a given promoter is activated or repressed could depend on the presence of homeodomain binding sites and the nature of the TATA box and on the relative activities of the various regulators acting on this promoter.

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